JTUSCI

Available online at www.taibahu.edu.sa

ISSN: 1658-3655

El-Naggar et.al / JTUSCI 3: 8-13 (2010)

Journal of Taibah University for Science

Biotechnology & Molecular Microbiology

Molecular diagnosis of *Candida albicans* using real-time polymerase chain reaction of a CaYST1 gene

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Received 29 June 2010; accepted 9 August 2010

Abstract

Candida albicans is an opportunistic pathogen that causes infections in immuno-compromised individuals. Virulence of this organism is a function of a multiplicity of factors working jointly to overwhelm the host defenses. From a previous study, the *in vitro* adherence capabilities of seven isolates (collected from patients suffering vaginitis) to vaginal epithelial cells were tested. Isolate No. 2 recorded the highest adherence ability. In this part of work, Real-time PCR assay was applied for the identification of isolate No. 2. This isolate could be discriminated by species-specific primers, deduced from the intron nucleotide sequence of the *C. albicans CaYST1* gene. To confirm that the isolated strain is a fungal strain, the universal fungal primers (ITS5 and ITS4) were used to amplify the complete internal transcribed spacer (ITS) region including the 5.8S ribosomal gene that is present in all fungi. The ITS region was successfully amplified from the tested yeast, provided a single PCR product of approximately 520 bp. In another run, a single pair of specific primers [Intron Nucleotide Sequence (INT1 and INT2)] was then used to amplify the intron nucleotide sequence of the *CaYST1* gene, which present only in *C. albicans*. The generated amplified product gave the expected single band of 310 bp. The melting points of all PCR products were routinely determined. The results showed that the melting temperatures for the tested isolate and the reference strain of *C. albicans* (ATCC 10231) using ITS5 and ITS4 primers were 80.09°C and 83.99°C, respectively, while they were 80.78°C and 80.10°C using INT1 and INT2 when assayed individually. Based on their very close melting profiles, the tested isolate proved to be identical to *C. albicans* reference strain. These results supported the idea of using genes containing intron sequences to design species-specific primers for the identification of fungal strains by real time PCR.

Keywords: *Candida albicans*, real-time PCR, *CaYST1* gene, INT1, INT2, ITS5, ITS4.

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1. Introduction

Fungal kingdom is incredibly diverse, and its members can inhabit an extraordinarily wide range of niches. Although some of these fungi are primary pathogens, there is a group of opportunistic fungi that can become pathogenic when the conditions in the host become favorable [1]. The yeast *Candida* is an example of a highly successful opportunistic pathogen that takes advantage of host debilities and disorders to cause infections of a remarkably wide range of tissues [2]. Identification of *Candida* species, at both medical and research laboratories, is often based on standard laboratory techniques such as germ tube formation [3] and biochemical tests [4-6]. These procedures are time-consuming, and have an inherent weakness in that they may not be species specific.

Real-time PCR is a useful tool for studying etiologic agents of infectious diseases [7, 8] and can be regarded as a method of choice due to its rapidity, sensitivity and reproducibility, where the risk of carry-over contamination is minimized. Although the conventional PCR has had a big impact with very good results in research, researchers have had difficulties due to the post-PCR steps for amplicon evaluation such as agarose gel electrophoresis. Real-time PCR allows the scientist to actually view the increase in the amount of DNA as it is amplified and post-PCR manipulation of the amplicon is not required, since the fluorescent signals are directly measured as they pass out of the reaction vessel. Furthermore, most of the real-time PCR components involve hybridization of oligoprobes to a complementary sequence of the amplicon strands [9].

Rapid identification of *Candida* species has become particularly important because of the increase in the number of infections caused by newly emerging species. The present study was conducted to identify locally isolated strain causing mycotic vaginitis using molecular approaches.

2. Materials & Methods

2.1. Microorganism and culture conditions

The yeast isolate was sub-cultured onto a set of three tubes of Sabouraud's dextrose broth (10 ml) that sequentially had 1, 2 and 3 drops of 1N HCl added to them. These broth cultures were incubated for 48-72 h at 25°C. A loop of the inoculated Sabouraud's dextrose broth (supplemented with 2 drops 1N HCl) was streaked on Sabouraud's dextrose agar plates, the plates were then incubated at 25°C for 48 h. [10] before genomic DNA extraction. To confirm the primers specificity, a reference strain (kindly provided by the Laboratory of Mycology, King Abdulaziz University Hospital, Jeddah, KSA) of *C. albicans* (ATCC 10231) was used.

2.2. Genomic DNA isolation

MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Biochemicals, Germany) was used with the MagNA Pure Compact Instrument to purify high-quality, undegraded genomic DNA from cultured yeast cells. The isolated DNA was eluted to meet the quality standards required for highly sensitive and quantitative PCR analysis on the LightCycler instrument [11].

The MagNA Pure Compact instrument automatically performs all isolation steps such as cell lyses, protein digest, binding of DNA, washing steps and elution of the pure nucleic acid. The pure elutes were stored at –20°C.

2.3. Primers

Two pairs of primers were used in this study (Table 1). The first pair designated as ITS5 and ITS4. This pair is universal fungal primers targeted the internal transcribed spacer (ITS) regions of rDNA [12]. The second pair of primers is designated as INT1 and INT2 (MWG Biotech, Ebersberg, Germany). This pair is specific primers deduced from the intron nucleotide sequence of the *C. albicans CaYST1* gene [13].

2.4. Real Time PCR reactions

Real-time PCR reactions were performed [14] using a Roche LightCycler 2.0 Instrument and LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Germany). The capillaries were transferred into the sample carousel of the LightCycler instrument and the samples were cycled. The program consisted of a denaturation step (95°C for 10 min), followed by 45 cycles of touchdown PCR (10 s at 95°C, 5 s at the annealing temperature listed in Table 1 and 10 s at 72°C) and a melting-curve step (50–98°C). Afterwards, cooling to 40°C for 30 s took place. The PCR process was monitored by fluorescence quantification of the DNA-binding dye SYBR green for detection of double-stranded amplified DNA and the melting analysis allowed identification of the species by a characteristic melting profile.

2.5. Agarose gel electrophoresis

The molecular sizes of the PCR products were determined by agarose gel electrophoresis in order to ascertain that only the expected products had been generated [15]. The most convenient method of visualizing DNA in agarose gel is by staining with a fluorescent dye as ethidium bromide. DNA-ethidium bromide complexes absorb ultraviolet light at 260, 300 or 360 nm and emit at 590 nm in the red-orange region of the visible spectrum. The gel was placed on the filler area of UV trans-illuminator (Spectroline – model TR-312A), then it was viewed and photographed.

Table 1. The sequences and LightCycler PCR conditions for each set of primers.

Primer Designation	Primer sequence (5'- 3')	LightCycler PCR conditions		Expected amplicon size (bp)
		$T_{Annealing}$ (°C)	$MgCl_2$ (mM)	
INT1-F	A A G T A T T T G G G A G A A G G G A A A G G G	55	3.0	310
INT2-R	AAAATGGGCATTAAGGAAAAGAGC			
ITS5-F	G G A A G T A A A A G T C G T A A C A A G G	65	1.5	520
ITS4-R	TCCTCCGCTTATTGATATGC			

3. Results & Discussion

The incidence of various fungal pathogens has increased dramatically over the past few decades. *Candida* species are the most common of these pathogens [4, 16]. Vulvovaginitis is one of the commonest presentations of *Candida* infection. The present study was conducted to identify the locally isolated *C. albicans* strains causing mycotic vaginitis.

Although conventional approaches are still valid and provide useful information for preliminary identification, many molecular biological developments have emerged with great promise for the detection of microbial pathogens as DNA-based molecular diagnosis. However, most of these molecular methods unfortunately require highly specific genetic information for a specific pathogen, which might not be always available.

In the present part, automated DNA extraction was performed and coupled with real-time PCR technique to identify the isolated strain No. 2. To confirm that the isolated strain is a fungal strain, the universal fungal primers (ITS5 and ITS4) were used to amplify the complete internal transcribed spacer (ITS) region including the 5.8S ribosomal gene that is present in all fungi. The ITS region was successfully amplified from the tested yeast, provided a single PCR product of approximately 520 bp. In another run, a single pair of specific primers (INT1 and INT2) was then used to amplify the intron nucleotide sequence of the *CaYST1* gene, which presents only in *C. albicans*. The generated amplified product gave the expected single band of 310 bp (Fig. 1).

To verify and confirm the accuracy of the identification, a reference strain of *C. albicans* (ATCC 10231) was used. DNA template was prepared from the broth culture and was amplified by PCR, and the sizes of the products were compared to the results of the isolate No. 2. In this case, the product generated from the tested clinical isolate was the same size as that generated by the corresponding reference strain (Fig. 2).

The melting points of all PCR products were routinely determined. The results showed that the melting temperatures for the tested isolate and the reference strain of *C. albicans* (ATCC 10231) using ITS5 and ITS4 primers were 80.09°C and 83.99°C, respectively, while they were 80.78°C and 80.10°C using INT1 and INT2 when assayed individually. Based on their very close melting profiles, the tested isolate was identical to *C. albicans* reference strain. The two pairs of primers used in this study successfully amplified their targets in the tested isolate.

More recently, real-time PCR techniques have been developed for the detection of fungal pathogens such as *Candida* species, *Cryptococcus neoformans* and *Aspergillus* species. The signal to be analyzed can be generated by double-stranded DNA-specific dyes, such as SYBR Green, or by sequence-specific fluorescence energy transfer probes [17]. Real-time PCR assays dramatically decrease the risk of false-positive results compared to the traditional PCR technique. Indeed, contamination with previously amplified products is reduced because the reaction tubes have to be closed following amplification, thus avoiding potential contamination of the environment with amplicons [18, 19]. The LightCycler system offers another advantage of analysis of the melting temperature T_m of amplicons. The melting temperature of the amplicon is dependent on the G+C content, sequence length and compositional variation in the nucleotide bases. Each fungal species has a characteristic T_m, which helps further to confirm its identity [17]. Therefore, this technology together with melting-curve analysis was used for the identification of *Candida* species [20].

On the other hand, the major limitation of PCR based diagnosis is the inhibition of the reaction caused by a variety of components within clinical specimens. The demand for PCR diagnosis in medical microbiology has highlighted the need for

efficient methods of nucleic acid extraction [21]. For this reason, a fully automated nucleic acid extraction technique with the MagNA Pure Compact Nucleic Acid Isolation Kit I was employed in this study. This apparatus can purify DNA from a broad variety of samples by magnetic bead technology, thus eliminating the need for vacuum pumps, centrifugation, or other manual steps that may result in cross contamination [22].

In the majority of eukaryotes, genes encoding rDNA and spacers occur in tandem repeats that are thousands of copies long, each separated by regions of non-transcribed DNA termed intergenic spacer (IGS) or non-transcribed spacer (NTS). Coding regions of the 18S, 5.8S and 28S nuclear rDNA genes are relatively conserved among fungi, which evolve slowly and provide a molecular basis of establishing phylogenetic relationships [23]. Between coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2, respectively) which evolve more rapidly and may therefore vary among different species within a genus. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic at the species level [24], and even within species (e.g. to identify geographic races).

In the present study, universal fungal primers were used to amplify the entire internal transcribed spacer region (ITS). The ITS region, located between the 18S and 28S ribosomal DNAs, is sub-divided into two spacers, ITS1 and ITS2, separated by the 5.8S conserved region. The ITS1 and ITS2 regions are highly variable sequences that have been used for identification of fungi at the species level [25-27]. The results obtained have shown that the primers successfully amplified the ITS region of the isolated yeast and provided a single PCR product of approximately 520 bp. However, identification of the amplicon is an important issue in this case and need an affordable procedure for characterization of the PCR products to the species level. Hybridization with species-specific DNA probes, using appropriate restriction enzymes, direct sequencing of the PCR product [23] or precise determination of lengths of PCR products [28-31] are time consuming methods and require expensive equipments that may not be readily available in many diagnostic laboratories.

A PCR technique to identify *C. albicans* and *C. dubliniensis* isolates, which uses species-specific primers from *EFB1* and *ACT1* intron sequences respectively, has recently been described. Maneu *et al.* [32] pointed out that the use of a single pair of primers, deduced from the intron and exon nucleotide sequences of the *C. albicans EFB1* gene with whole cells of both laboratory strains and clinical isolates of *Candida* species, resulted in the species-specific amplification of a 785 bp DNA fragment in *C. albicans* strains.

Therefore, the use of primers deduced from the *C. albicans CaYST1* gene intron was described to specifically amplify a DNA fragment in *C. albicans* strains. The *CaYST1* gene codes for a protein found in membrane and ribosome fractions. It is a component of the translational machinery and well conserved among species [33]. However, the advantage of the use of this sort of primers is to save additional manipulation of the PCR product(s). There is no need to use multiple primer pairs and nested PCR, restriction enzyme digestion, or DNA sequencing to differentiate the *Candida* species present in the sample. So, the simple approach used in this study, incorporated just one pair of primers to detect medically isolated *C. albicans*, since the intron sequences are poorly conserved among other strains, this 310 bp amplicon was used and designated as *CaYST1*-INT, to identify *C. albicans*.

The results obtained by this molecular assay revealed that primers INT1 and INT2 designed for the specific identification of *C. albicans* produced the expected 310 bp amplicon from the tested isolate and the reference strain. The results in this study indicated that genes containing intron sequences could well be useful to design specific primers for direct identification of *Candida* at the species level, which could be useful in earlier detection of candidiasis. Thus, the primer set identified the tested isolate as *C. albicans*; this strengthens the result obtained by the conventional methods and API technique. These results are in agreement with Baquero *et al.*[13] who identified *C. albicans* by PCR amplification of the *CaYST1* gene intron fragment. The PCR analysis was performed using both genomic DNA and whole cells of the clinical isolates of *Candida* species. They found that all the clinical *C. albicans* isolates generated the expected 310 bp amplicon.

4. Conclusion

In conclusion, when the primers deduced from *Candida* gene intron are used, these PCR approaches would be very beneficial from a prognostic and therapeutic point of view and will be helpful for epidemiological and taxonomic issues.

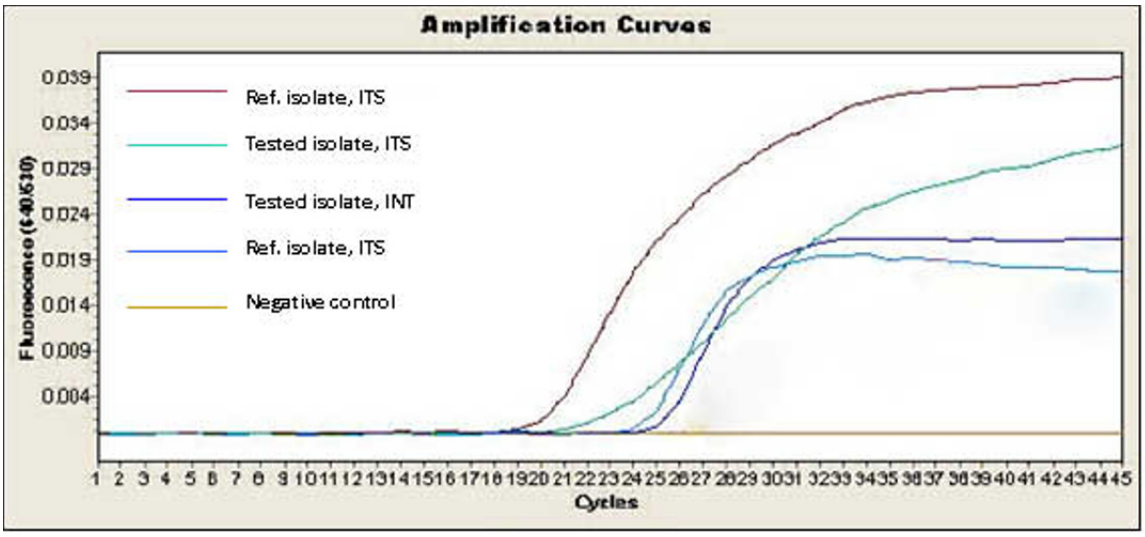

Fig.1. Amplification curves of the tested isolate and *C. albicans* ATCC 10231 in LightCycler PCR using ITS5, ITS4, INT1 and INT2 primers. As a negative control, template DNA was replaced by PCR-grade water.

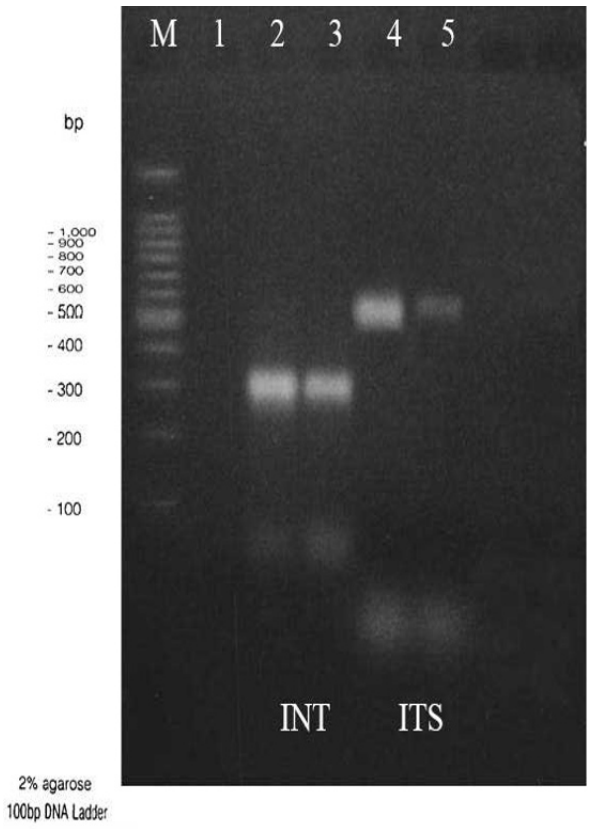

Fig.2. Electrophoretic pattern of LightCycler-amplified DNA products. Lane M, 100-bp marker; Lane 1, negative control, no DNA template (distilled water); Lane 2, *C. albicans* ATCC 10231 (310 bp); Lane 3, the tested isolate (310 bp); Lane 4, *C. albicans* ATCC 10231 (520 bp); Lane 5, the tested isolate (520 bp).

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